# Transfer of Amphotericin B from Gel State Vesicles to Mycoplasma Cells: Biphasic Action on Potassium Transport and Permeability

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The action of amphotericin B on the $K^+$ permeability of *Mycoplasma mycoides* var. *capri* cells, containing either cholesterol or ergosterol in their membranes, was studied. When the drug, solubilized in dimethyl sulfoxide, was added directly to the cell suspension, a slightly greater sensitivity to permeabilization was observed for ergosterol-containing cells, confirming the data reported in the literature. When amphotericin B bound to gel state phospholipid vesicles was added to the cell suspension, two effects on cholesterol-containing cells were observed. First, the $K^+$ active transport rates increased; membrane permeabilization and $K^+$ leakage were subsequently detected. For ergosterol-containing cells these sequential events were observed only at amphotericin B concentrations below $10^{-6}$ M. At higher concentrations only $K^+$ leakage was observed. The second permeabilization effect varied with the amphotericin B concentration in different ways in the two types of cells. The permeabilization of ergosterol-containing membranes depended on the amphotericin B/phospholipid molar ratio, whereas the permeabilization of cholesterol-containing membranes did not. In general, the latter remained fairly constant when the total amphotericin B concentration in the medium varied.

The antibiotic activity of amphotericin B (AmB) is related to the channels this drug forms in cell membranes (for a review, see reference 11). AmB and related compounds are used in antifungal therapy, since ergosterol-containing membranes are more sensitive to their action than cholesterol-containing ones (for a review, see reference 15). Although numerous studies have been carried out, leading to the development of elaborate models (9, 17), little is known about the complex process which occurs during the interaction of AmB with membranes and which leads to pore formation.

The poor water solubility of AmB, which results in its aggregation in aqueous medium, has been a major impediment in studying this process. The interaction of these aggregates with membranes is difficult to analyze in terms of the successive molecular events involved and thus has been dealt with as an all or nothing process.

This impediment can be overcome by making use of the ability of AmB to exchange between membranes: instead of introducing into the cell suspension AmB as a dimethyl formamide or dimethyl sulfoxide (DMSO) solution, which results in its aggregation, AmB is first incorporated into lipid vesicles which are then introduced into the membrane suspension. By this method, which completely eliminates the presence of AmB in its aggregated form, it has been shown that the affinity of AmB for membranes is a function of the physical state of the membrane (6). More recently, a study of the interaction of AmB with cholesterol and ergosterol-containing phospholipid vesicles revealed that the selectivity of AmB for the latter was much greater than the selectivity currently admitted on the basis of the results obtained by direct application to these membranes of the antibiotic dissolved in organic solvent (19).

In this report we present the results of a study of the interaction of AmB, either dissolved in DMSO or incorporated into small sonicated phospholipid vesicles

(SUV), with *Mycoplasma* cell membranes. This organism, grown in the presence of ergosterol or cholesterol, has been used as a model membrane system for fungal and animal cells, respectively (1). The sensitivity of the two types of cell membranes appears to be widely different. Furthermore, using these milder conditions of interaction with the membrane, we unmasked an activity of AmB on the active transport system of the cell membrane that is quite different from that of permeabilization.

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## MATERIALS AND METHODS

**Materials.** AmB was a generous gift from E. R. Squibb & Sons (France), and L-α-dipalmitoylphosphatidyl choline (DPPC) was obtained from Sigma Chemical Co. Ergosterol and cholesterol were from Fluka and Merck, respectively. Both were purified by double recrystallization in ethanol before use. Dimethyl sulfoxide (DMSO) was obtained from Prolabo (France).

**Organism.** *Mycoplasma mycoides* var. *capri* PG3 was grown by the method of Leblanc and Le Grimellec (14). Modified Edward medium was supplemented with 0.5% fatty acid-poor albumin (fraction V; Pentex), 5 mg each of oleic acid and palmitic acid per ml, and 0.1 mg of cholesterol per ml. These cells (PG3-C) were adapted to ergosterol by repetitive transfer, twice a day, in the same culture medium, containing 0.1 mg ergosterol per ml instead of cholesterol. After 2 weeks, these cells (PG3-E) were adapted and could be grown like the normal PG3-C cells. Although they grew more slowly, PG3-E cells behaved quite similarly to PG3-C cells (1, 2, 14).

Experiments were carried out with the two types of cells harvested by centrifugation (8,000 × *g*, 10 min) during the linear growth phase (absorbance of 0.27 at 640 nm), that is, with young cells homogeneous in size and shape. Cells were washed in cold hypertonic medium (250 mM NaCl–10 mM
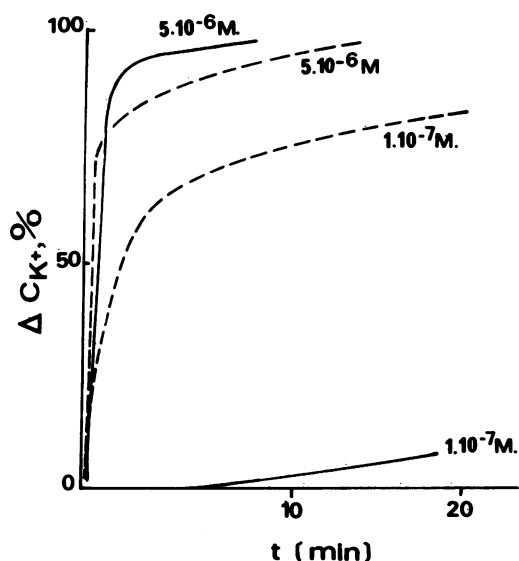
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FIG. 1. $K^+$ efflux induced in PG$_3$-C (——) and PG$_3$-E (----) cells by the direct addition of AmB at the indicated concentration (at 27°C; cell concentration, 1 mg of protein per ml). $K^+$ effluxes are expressed as $\Delta C_{K^+}$, the variation of the $K^+$ external concentration as the percentage of the maximum variation obtained after cell lysis by digitonin. $t$, Time.

MgCl$_2$) and used the same day. By using a standard curve, the protein content was determined by measuring the optical density at 640 nm.

**SUV preparations.** SUV were prepared by dissolving weighed amounts of DPPC in chloroform in a round-bottomed flask. After removal of the solvent in vacuo, the dry film was blended with a Vortex mixer, in the desired volume of buffer used as the suspension medium of the cells in the experiment (see below). The lipid suspension was then sonicated to clearness under nitrogen (above the transition temperature) and then centrifuged (2,000 × g, 10 min) for removal of metal particles and undispersed lipids.

**Measurement of $K^+$ efflux.** Freshly collected cells at a concentration of 1 mg of protein per ml were incubated 10 min at 37°C in the following buffer: 100 mM sodium phosphate, 40 mM sodium chloride, 2 mM magnesium chloride, 100 mM glucose (pH 7.2). After incubation, 2 ml of this cell suspension was transferred into the experimental vial and equilibrated at 27°C until the $K^+$ concentration ($10^{-4}$ M in this condition) was stabilized in the suspension medium. Then, AmB was added, as described below, and the $K^+$ concentration was continuously monitored in the suspension medium by a $K^+$-sensitive electrode (type F 2312 K Radiometer; Copenhagen, Denmark). At the end of the experimental period, 20 μl of a $10^{-2}$ M solution of digitonin in ethanol was added, and the total $K^+$ concentration was measured.

AmB was introduced by the following methods. (i) By the direct method, AmB was added to the cell suspension as microliter amounts of a freshly prepared $10^{-3}$ M solution in DMSO. (ii) By the transfer method, AmB was added to the cell suspension of 100 μl of a suspension of DPPC-SUV (corresponding to 2 mM final lipid concentration). SUV were incubated with the desired amount of AmB until the specific circular dichroism signal of the aggregated form of the drug in aqueous solution completely disappeared. Thus, whatever the quantity of AmB present in the SUV suspension, its

concentration in the aqueous phase was lower than its critical micellar concentration (CMC) (<$10^{-7}$ M). As a consequence, the amount of AmB in the aqueous phase was negligible compared with that in the membrane phase (18).

## RESULTS

**Direct method.** A typical time course of the $K^+$ efflux induced by two different concentrations of AmB (5 × $10^{-6}$ and $10^{-7}$ M) on PG$_3$-C and PG$_3$-E cells is given in Fig. 1. The $K^+$ efflux is expressed as a percentage of the maximal $K^+$ concentration reached in the medium after complete lysis by digitonin (4). The kinetics of $K^+$ efflux are not exponential but are composed of a rapid phase followed by a much slower one, which is observed only at very low antibiotic concentrations (<$10^{-7}$ M, below the CMC). PG$_3$-E cells appear to be more sensitive to AmB than do PG$_3$-C cells. In Table 1 are given the times necessary to obtain a $K^+$ leak corresponding to 50% of the cell content. It should be stressed that since the $K^+$ leak is not exponential, this value cannot be taken as a $t_{1/2}$ value, but only as a convenient index for comparison. The smaller the AmB concentration, the greater the difference appeared between cholesterol- and ergosterol-containing membranes.

**Transfer method.** The results of the transfer method are given in Fig. 2A (PG$_3$-C) and 2B (PG$_3$-E). The action of AmB transferred from DPPC-SUV to *Mycoplasma* cells resulted in a biphasic change of the external $K^+$ concentration, which first decreased then increased. In the experimental conditions chosen (the cells were in an energized state in the presence of glucose), the constancy of $K^+$ concentration in the absence of antibiotic was the result of a steady-state equilibrium between $K^+$, the energy-dependent intake, and $K^+$ leakage. Therefore, since on AmB addition the external $K^+$ concentration decreased, the energized rate of $K^+$ intake by the cells was faster than the rate of $K^+$ leak.

The changes of $K^+$ concentration in the suspension medium presented in Fig. 2A and B can be accounted for by two opposite effects: a nearly instantaneous (within 1 to 2 s) activation of $K^+$ intake followed by a much slower development of the $K^+$ leak through channel formation.

The activation effect is not due to the DPPC-SUV themselves, since DPPC-SUV did not induce any significant change of external $K^+$ concentration during the same experimental period (data not shown). If the addition of DPPC-SUV was followed by the addition of AmB in DMSO solution, the changes in the external $K^+$ concentration were identical to those obtained by adding SUV preloaded with AmB (data not shown); this meant that when the antibiotic was added to a mixture of *Mycoplasma* cells and DPPC-SUV it bound first to the vesicles, which is consistent with the much greater affinity of the antibiotic to lipid membranes in the gel state (6). Moreover, it is worth noting that this paradoxical activation effect was never observed by the

TABLE 1. Effect of AmB direct addition on $K^+$ leakage

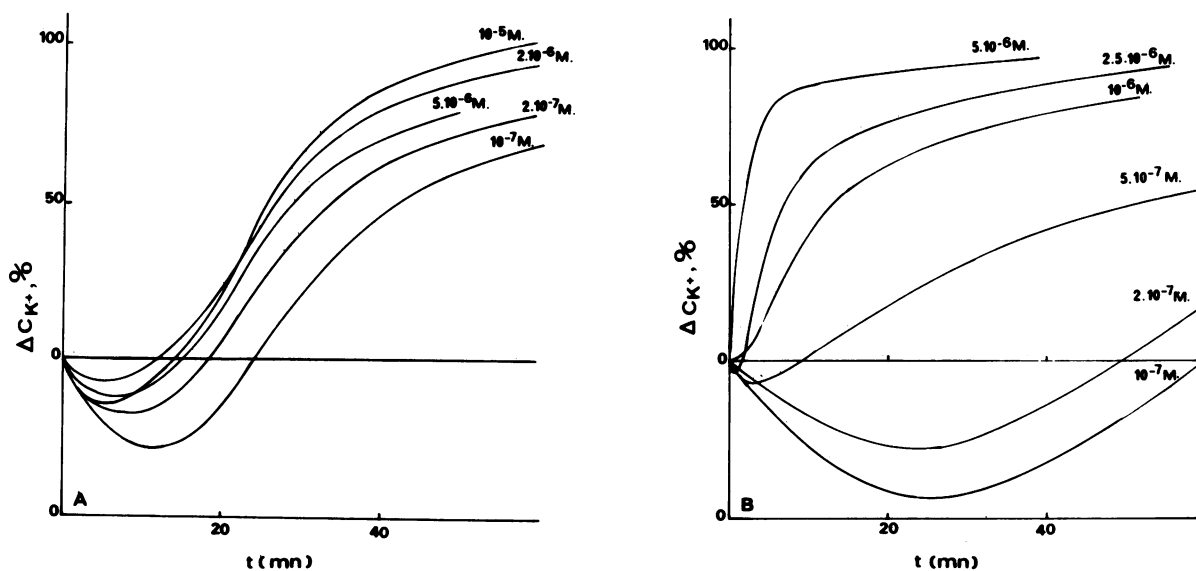| AmB conc (×$10^6$ M) | Times for 50% $K^+$ leakage in the following cells: | |
|---|---|---|
| | PG$_3$-E | PG$_3$-C |
| 0.2 | 3 min | >80 min |
| 2.0 | 1–2 min | 4 min |
| 5.0 | 10–40 s | 50 s |
| 10.0 | ~1 s | 5–10 s |

FIG. 2. $K^+$ fluxes induced in $PG_3$-C (A) and $PG_3$-E (B) cells by AmB incorporated in DPPC vesicles (at 27°C; cell concentration, 1 mg of protein per ml); concentration of DPPC, 2 mM. $K^+$ fluxes are expressed as $\Delta C_{K^+}$, the variation of the $K^+$ external concentration as the percentage of the maximal variation obtained after cell lysis by digitonin. $t$, Time (expressed in minutes [mn]).

direct addition of AmB even at a very low concentration (below the CMC, $10^{-7}$ M; see Fig. 1).

The effect of the AmB concentration was studied at constant DPPC concentration by increasing the AmB/DPPC molar ratio. The effects on $PG_3$-C and $PG_3$-E were very different. In the first case (Fig. 2A), within the range studied ($10^{-5}$ to $10^{-7}$ mol of AmB per liter of suspension), $K^+$ flux varied little. Above $10^{-7}$ M, the amplitude of the primary effect in $K^+$ intake decreased with increasing AmB concen-

tration. This was probably due to the increasing slowness and weakness of the secondary ionophoric effect of $K^+$ leakage inducement. The ascendent part of the curves, corresponding to this latter effect, appeared to have comparable amplitudes throughout the AmB concentration range. The time necessary to obtain a $K^+$ leak corresponding to 50% of the cell content varied from 25 min at $10^{-5}$ M to about 45 min at $10^{-7}$ M.
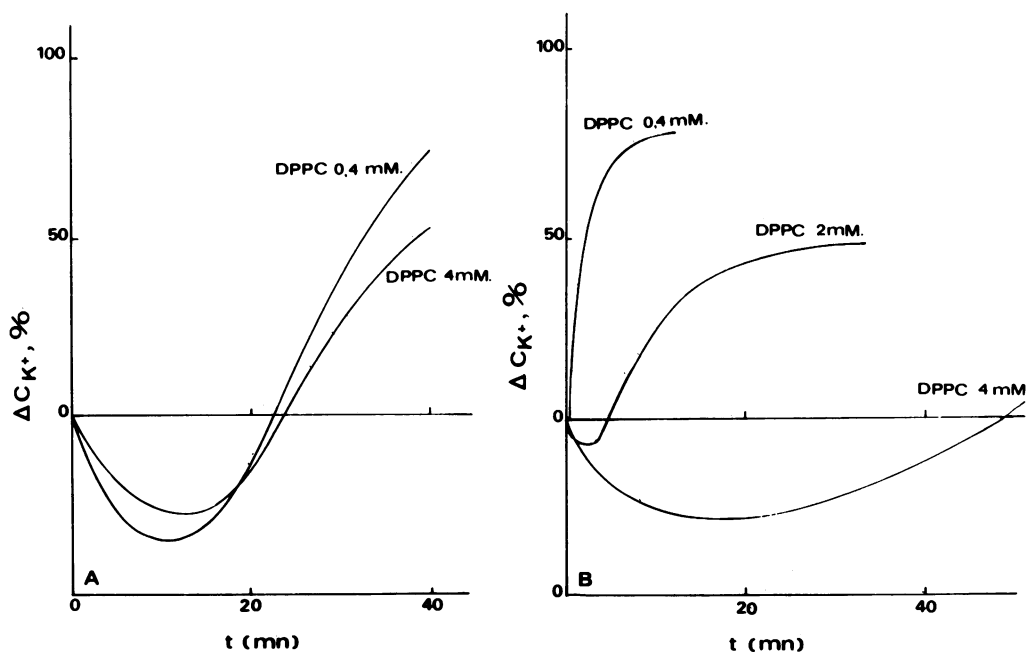
$PG_3$-E cells were more sensitive to variations in the AmB

FIG. 3. $K^+$ fluxes induced in $PG_3$-C (A) and $PG_3$-E (B) cells by the addition of a constant concentration of AmB ($5 \times 10^{-7}$ M) incorporated in DPPC vesicles at various AmB/DPPC molar ratios. The corresponding DPPC concentrations are indicated on the curves (temperature, 27°C; cell concentration, 1 mg of protein per ml); $K^+$ fluxes are expressed as $\Delta C_{K^+}$, the variation of external $K^+$ concentration as the percentage of the maximal variation obtained after cell lysis by digitonin. $t$, Time (expressed in minutes [mn]).

concentration in the DPPC-SUV. The primary effect was completely canceled out by the ionophoric effect at $5 \times 10^{-6}$ M, whereas at $10^{-7}$ M the primary effect was still observed even after 60 min.

In these experiments the concentration dependencies involved two parameters: the antibiotic concentration in the suspension and the molar ratio of AmB/DPPC. To check the latter, experiments were carried out at constant AmB concentrations, but variable DPPC concentrations. DPPC-SUV were loaded at various AmB/DPPC molar ratios and added to the cell suspension such that the antibiotic concentration in moles per liter of suspension was kept constant. In Fig. 3A ($PG_3$-C) and B ($PG_3$-E) are shown the results obtained at a constant AmB concentration of $5 \times 10^{-7}$ mol/liter of suspension and at three different AmB/DPPC molar ratios: $12.5 \times 10^{-4}$, $2.5 \times 10^{-4}$, and $1.25 \times 10^{-4}$. The value of this ratio was without influence for $PG_3$-C cells, whereas it was very important for $PG_3$-E cells, in which the large dependency observed in Fig. 2B on the AmB concentration was possibly due to the AmB/DPPC molar ratio variation.

## DISCUSSION

The first part of this study, dealing with the direct action of AmB on *Mycoplasma* cells grown either on cholesterol- or ergosterol-containing medium, was performed to provide results comparable to those obtained by AmB transfer from DPPC-SUV. The higher sensitivity observed for ergosterol-grown cells is consistent with the results obtained by Archer for the interaction of the same types of cells with AmB-methyl ester, a chemically modified AmB molecule (2). On the other hand, similar biphasic kinetics were consistently observed when AmB was introduced in DMSO solution to pure lipidic vesicles (either SUV or large unilamellar vesicles). For these model systems $^{31}$P-NMR spectroscopy has shown (unpublished data) that upon AmB addition, two populations of vesicles can be distinguished: a population immediately and massively permeabilized (probably by direct interaction with antibiotic aggregates) and a population permeabilized much more slowly (probably by a secondary exchange of AmB between vesicles). It may be assumed that similar processes occur with *Mycoplasma* cells.

A novel result appears in the second part of our study, where AmB transferred from DPPC-SUV to *Mycoplasma* cells. Besides inducing permeability (as observed by its direct addition), this polyene also produced a quite different effect on the membrane active transport mechanisms, namely the uptake of $K^+$. Various activation effects of AmB applied at sublethal doses have already been observed in various biological systems. Some of these effects are considered to be consequences of the perturbation of the cell membrane passive permeability (5, 16), and others are attributed to direct stimulation of active transport (7, 8, 12). It appears to be difficult to relate the transitory net potassium intake observed in the present experiments to a change in passive permeability. A mechanism involving active transport would seem to be involved. The $K^+$ transport in *M. mycoides* var. *capri* has been studied extensively by Benyoucef et al. (3, 4). These authors have shown that $K^+$ is transported by an active $Na^+$-dependent and ATP-consuming mechanism. How can AmB-loaded vesicles modulate this system? The conditions in which the present experiments were carried out correspond to a maximally energized state of the *Mycoplasma* cells, and therefore it is difficult to continue to increase it. On the other hand, the increase in $K^+$ intake arises within a few seconds on the addition of AmB-

loaded DPPC-SUV. Therefore, a mechanism involving, for instance, an increase of intracellular ATP production due to a massive intake of glucose is unlikely.

The pores formed by AmB allow $K^+$ leakage but also $Na^+$ intake since they exhibit practically no intercationic selectivity (11). In the presence of an excess of intracellular $Na^+$, the $K^+$ transport system could be stimulated, as already has been observed in AmB-treated 3T3 cells (16), but this stimulation would not lead to the net $K^+$ influx observed here. Finally, the simplest hypothesis seems to assume an activation of one of the systems involved in the active transport of $K^+$.

The mechanism by which AmB is transferred to the cells is still unknown. Results of experiments involving the transfer of AmB among lipidic vesicles suggest that fusion between DPPC and egg yolk lecithin-sterol vesicles does not occur even under AmB influence, but there is some indication of a transfer mechanism through the aqueous phase (6). However, this may not be true for the interaction between *Mycoplasma* cells and DPPC-SUV. Grant and McConnell have shown that fusion of pure DPPC vesicles and *Acholeplasma laidlawii* cells is a massive process achieved within 30 min (10). In the present case, if such a massive fusion occurs (50% of the total vesicle content), the correlated incorporation to the intracellular medium of a non-negligible amount of external medium would certainly lead to a change of the $K^+$ balance even in the absence of AmB. Actually, this does not occur. Furthermore, under the conditions used in this study, stimulation is observed within a few seconds after AmB-loaded vesicles are added.

Experiments carried out on *A. laidlawii* indicated that these cells reacted similarly to $PG_3$ (data not shown). Fusion between DPPC vesicles and cells has been demonstrated in other cases, in particular with *Acanthamoeba castellanii* (13). Fusion or endocytosis after adsorption at the cell surface could be the mechanism of AmB transfer. AmB transfer therefore may occur by direct contact between membranes.

Whatever the transfer mechanism is, the sensitivity to AmB, observed indirectly, is very different for ergosterol-containing than for cholesterol-containing membranes. This difference seems to concern mainly pore formation and $K^+$ leakage. At a high AmB/DPPC ratio and a relatively low molar concentration of the antibiotic in the medium, ergosterol-grown *Mycoplasma* cells appear to be 10 times more sensitive to the permeabilizing action of AmB than cholesterol-grown cells. However, the contrary result is obtained when the concentration of the antibiotic in the medium is relatively high. This observation may be a clue to understanding the process of pore formation in the two types of membranes and might be of some help in designing a better strategy for AmB systemic administration in antifungal therapy. That AmB might act directly on the mechanism of active transport offers an alternative hypothesis concerning the mechanism by which AmB is able to potentiate or stimulate a number of other membrane processes.

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